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EXPERIMENTAL ARTICLES

Diversity of Diazotrophic Gut Inhabitants of Pikas (*Ochotonidae*) Revealed by PCR-DGGE Analysis[1]

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Abstract—Diazotrophic gut symbionts are considered to act as nitrogen providers for their hosts, as was shown for various termite species. Although the diet of lagomorphs, like pikas or rabbits, is very poor in nitrogen and energy, their fecal matter contains 30–40% of protein. Since our hypothesis was that pikas maintained a diazotrophic consortium in their gastrointestinal tract, we conducted the first investigation of microbial diversity in pika guts. We obtained gut samples from animals of several *Ochotona* species, *O. hyperborea* (Northern pika), *O. mantchurica* (Manchurian pika), and *O. dauurica* (Daurian pika), in order to retrieve and compare the nitrogen-fixing communities of different pika species. The age and gender of the animals were taken into consideration. We amplified 320-bp long fragments of the *nifH* gene using the DNA extracted directly from the colon and cecum samples of pika's gut, resolved them by DGGE, and performed phylogenetic reconstruction of 51 sequences obtained from excised bands. No significant difference was detected between the nitrogen-fixing gut inhabitants of different pika species. NifH sequences fell into two clusters. The first cluster contained the sequences affiliated with NifH Cluster I (Zehr et al., 2003) with similarity to *Sphingomonas* sp., *Bradyrhizobium* sp., and various uncultured bacteria from soil and rhizosphere. Sequences from the second group were related to *Treponema* sp., *Fibrobacter succinogenes,* and uncultured clones from the guts of various termites and belonged to NifH Cluster III. We suggest that diazotrophic organisms from the second cluster are genuine endosymbionts of pikas and provide nitrogen for further synthesis processes thus allowing these animals not to be short of protein.

Keywords: nifH, nitrogen fixation, pikas, *Ochotona*, gut microflora

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Animals have various food preferences, and many of them are herbivores, obtaining energy and nutrients from plant material. Two aspects of herbivore diet have always been of interest: cellulose digestion, since herbivores do not have enzymes for cellulose breakdown [1], and protein synthesis, since plants are low in proteins. Ruminants, such as cattle, rely on symbiotic microflora for cellulose digestion and utilize microbial protein synthesized in a special part of their four-chambered stomach called rumen [2]. Protein synthesis in the rumen is a complex process which involves organisms belonging to *Bacteria, Protozoa* and *Fungi* [3]. During this process, dietary protein and non-protein nitrogen are converted to ammonia and amino acids by microbes, which use these compounds to synthesize bacterial protein, partly transformed by ruminants into the body tissue protein and partly excreted with faeces [4].

Termites feed on various substrates, including wood, which is as much as 50 per cent cellulose [5], soil [6] and plant litter, as well as fungi cultivated in fungal gardens for further consumption. Cellulose consumed by the termites is usually broken by either cellulolytic protozoa, or by endogenous cellulases [7]. Symbiotic microflora of the termites provides them with nitrogen by means of nitrogen fixation, since termites' diet is usually very poor in this essential nutrient [8]. Diazotrophic symbionts of the termites are quite diverse and mostly belong to *Bacteria* [9] and methanogenic *Archaea* [10].

Mammalian herbivores face several challenges when consuming plant material as a primary food source. An order of mammals, known as *Lagomorpha* and comprising hares, rabbits, and pikas, combines the feeding mechanisms of ruminants and termites. Lagomorphs feed on grass and tree branches, but they have no rumen for fermentation purposes. Representatives of all families of *Lagomorpha* were shown to get protein into diet by means of caecotrophy, a form of coprophagy [11]. Lagomorphs have two types of faeces–solid faeces containing undigested food and soft faeces, rich in bacterial protein [12]. These mammals consume soft faeces, thus supplying their bodies with

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Table 1. Sequences of primers used in this study

Primer	Primer sequence (5'-3')	Target microbes	Reference
F1	TAYGGIAARGGIGGIATIGGIAARTC	N-fix *Bacteria* and *Archea*	[37]
R6	GCCATCATYTCICCIG	N-fix *Bacteria* and *Archea*	[37]
nifHfor	TAYGGNAARGGNGGHATYGGYATC	N-fix *Bacteria*	[38]
nifHrev	ATRTTRTTNGCNGCRTAVABBGCCATCAT	N-fix *Bacteria*	[38]
nifH-f	GGHAARGGHGGHATHGGNAARTC	N-fix *Bacteria*	[39]
nifH-r	GGCATNGCRAANCCVCCRCANAC	N-fix *Bacteria*	[39]
PolF	TGCGAYCCSAARGCBGACTC	N-fix *Bacteria*	[17]
PolR	ATSGCCATCATYTCRCCGGA	N-fix *Bacteria*	[17]
PolFI	TGCGAICCSAAIGCIGACTC	N-fix *Bacteria*	[18]
AGER-GC30	CGCCCGCCGCGCCCCGCGCCCGGCCCG CCCGACGATGTAGATYTCCTG	N-fix *Bacteria*	[18]
nifH-F	AAAGGYGGWATCGGYAARTCC ACC AC	N-fix *Bacteria*	[40]
nifH-R	TTGTTSGCSGCRTACATSGCCATCAT	N-fix *Bacteria*	[40]

Modied bases: I = Inosine, Y = CT, S = CG, R = AG, B = GCT, W = AT; N = ACGT; H = ACT.

protein. The dry substance of soft faeces of hares was shown to contain up to 39% of protein [13]; for rabbits this value was lower—up to 32%. The estimated protein content in the food of these animals is between 6 and 13%. Pikas appear to be much more efficient digesters of low-nutrient and low-protein food. They eat legumes and tree branches, which are 2–14 times less rich in protein than their soft faeces that contained 41–48% of protein [13].

Since the staple food of pikas is extremely poor in nitrogen, these animals probably obtain additional nitrogen by ways similar to those of the termites, i.e. via the symbiotic nitrogen fixation. Although the idea of symbionts involved in nitrogen utilization by herbivorous mammals has appeared earlier in the literature [14], nothing is currently known about the organisms inhabiting pika's gut and fixing nitrogen for further utilization of this essential nutrient for protein synthesis. In this study, we concentrated on the diversity of the *nifH* gene encoding nitrogenase reductase found in the intestines of several species of *Ochotonidae* family—*Ochotona hyperborea, O. mantchurica,* and *O. dauurica*. *NifH* is the marker gene which encodes a component of the evolutionary conserved key enzyme, responsible for the process of atmospheric nitrogen fixation. It is widely used for assessing the diversity of nitrogen-fixing communities in various habitats [15].

MATERIALS AND METHODS

Sample preparation. All gut samples were obtained at the Faculty of Biology of the Lomonosov Moscow State University. Two individuals of northern pikas *Ochotona hyperborea,* young male and female were collected during field work of 2009 and 2010 in Zabaykalsky Krai, Transbaikalia, Russia. One Manchurian pika *Ochotona mantchurica scorodumovi* [16] also originated from Transbaikalia. The gender of this pika was unknown. Two Daurian pikas *Ochotona dauurica* (a very young male and a sub-adult female) arrived to MSU after fieldwork of 2010 in the Daursky State Natural Biosphere Reserve Park, Siberia, located between two large lakes, Barun-Torey and Zun-Torey. For each animal, except for Manchurian pika, samples for analysis were taken from back end of the cecum, initial portion of the colon immediately distal to the cecum, and the distal colon, and tissue samples were stored in ethanol-containing buffer prior to analysis. For Manchurian pika, only the cecum sample was obtained. Food remains were not removed from the guts before DNA extraction.

DNA extraction and PCR amplification. DNA was extracted from the samples of pika intestines in 2010 and 2011 by means of Power Soil DNA Extraction Kit (MO BIO, United States) according to the manufacturer's recommendations with an obligate bead-beating step. Extracted DNA was used for PCR amplification of *nifH* gene. PCR was run in triplicates, and the products were pooled prior to further analysis. We tested several primer systems for *nifH* gene amplification, running PCR according to the authors' recommendations (Table 1), until obtaining a PCR product of expected size with PolF-PolR primers [17]. We used nested PCR approach, when a product of the first amplification round was used as a template for the second amplification round with primers Pol FI/AQER-GC30 [18]. *Bradyrhizobium japonicum* was chosen as a positive control strain for *nifH* amplification for both rounds of PCR. The primer AQER-GC30 had a GC-clamp attached to its 5' end for further separation of amplification products by means of denaturing gradient gel electrophoresis (DGGE) [19]. The PCR mixture for both amplification steps contained 1× PCR

Table 2. Description of samples resolved on DGGE lanes

Lane ID	Organism, gender	Age, sampling year	Part of gut
om	Manchurian pika, unknown	Unknown, 2009	Cecum
oh1	Northern pika, male	Juvenile, 2009	Cecum
oh2	Northern pika, male	Juvenile, 2009	Initial colon
oh3	Northern pika, male	Juvenile, 2009	Distal colon
oh4	Northern pika, female	Sub-adult, 2010	Cecum
oh5	Northern pika, female	Sub-adult, 2010	Initial colon
oh6	Northern pika, female	Sub-adult, 2010	Distal colon
od1	Daurian pika, male	Juvenile, 2010	Cecum
od2	Daurian pika, male	Juvenile, 2010	Initial colon
od3	Daurian pika, male	Juvenile, 2010	Distal colon
od4	Daurian pika, female	Sub-adult, 2010	Cecum
od5	Daurian pika, female	Sub-adult, 2010	Initial colon
od6	Daurian pika, female	Sub-adult, 2010	Distal colon

buffer (17 mM $(NH_4)SO_4$, 67 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, Helicon, Russia), 0.5 mM of each dNTP, 25 pmol of the forward and reverse primers, 1 μL of DNA template, 20 mg of bovine serum albumin (Fermentas, Canada), 5% of dimethyl sulfoxide (DMSO) and 1.25 U of *Taq* DNA polymerase (Helicon, Russia). The temperature protocol for both rounds of *nifH* amplification was as follows: 15 min of initial denaturation at 95°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final extension step was performed at 72°C for 10 min. Amplification was performed on MyCycler thermocycler (BioRad, United States). Amplification products were checked by electrophoresis in 1.2% agarose gel, stained by ethydium bromide.

DGGE analysis and sequencing. Denaturing gradient gel electrophoresis (DGGE) was performed on a D-Code Universal Mutation Detection System (Bio-Rad, United States) at a constant temperature of 60°C and constant voltage of 100 V for 16 h with a 40–65% range of denaturing agents (formamide and urea). After electrophoresis the gel was stained with ethydium bromide for 30 min and washed in sterile distilled water for the same amount of time as staining. The most distinct bands were excised, and DNA was eluted from the gel by soaking in sterile MQ water for at least 24 h at 4°C. Eluted DNA was used for reamplification with the primers PolFI and AQER without GC clamp. Amplification products were gel-purified by means of the QIAquick Gel Extraction Kit (Qiagen, Germany) and sequenced in the Bioengineering Center, Russian Academy of Sciences with help of Big Dye Terminator v.3 reagent kit (Applied Biosystems Inc., United States) and ABI Prism3100 automatic sequencer (Applied Biosystems Inc., United States).

Phylogenetic analysis. The NifH sequences obtained from the DGGE bands were compared with the sequences available in the GenBank database using the Basic Local Alignment Search Tool, BLAST at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). Nucleotide sequences were aligned and translated by means of the BioEdit software [20] and alignments were manually refined. Phylogenetic analyses were performed by applying neighbor-joining method using the respective tools implemented in the TREECON software package [21]. The significance of the nodes was measured by bootstrap analysis of 100 resamplings.

Nucleotide sequence accession numbers. All NifH sequences included into phylogenetic reconstruction in this study are available at GenBank under accession numbers JN416586–JN416598 and JQ928191–JQ928230.

RESULTS

Several degenerate *nifH*-targeted primer systems, which showed broad specificity in the environmental studies, were tested (Table 1), until a widely used primer system PolF/PolR, [17] gave a product of the expected length with all DNA samples extracted from the cecum and colon of pikas in the second PCR round. The *nifH* gene fragments were retrieved from the guts of all pika species, no matter whether the animal was male or female, young or adult. PCR with other primer pairs either gave non-specic primer binding resulting in several amplicons, or failed.

Generated PCR products showed stable and reproducible DGGE profiles (Table 2). Examination of DNA band profiles on the DGGE gel revealed that diazotrophic communities of different parts of the gut produced distinct banding patterns with different electrophoretic positions of the bands (Fig. 1). A wide va-

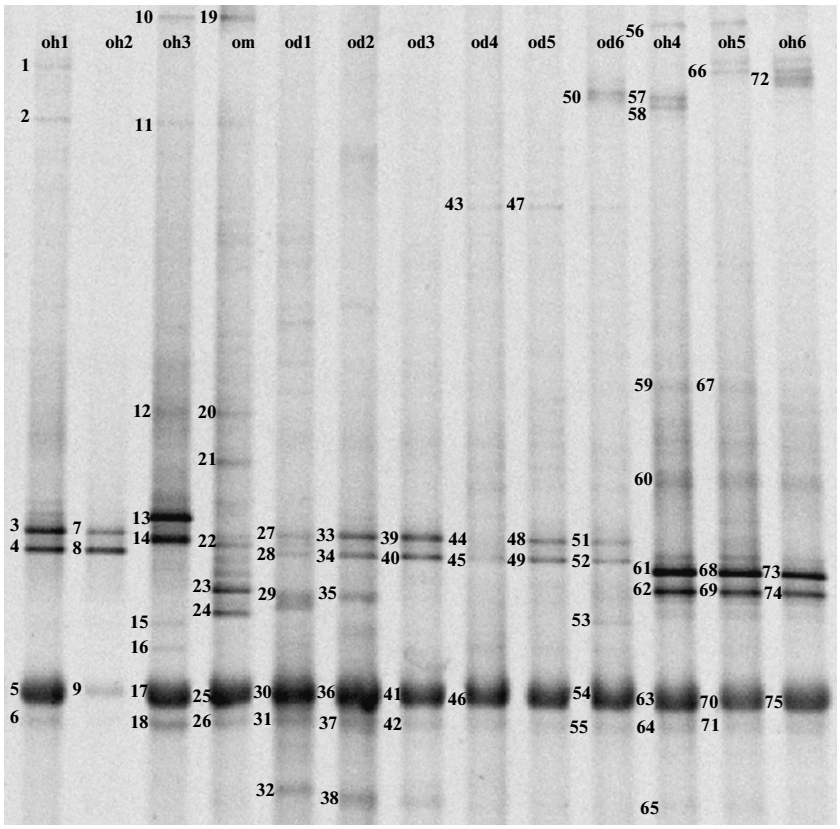

Fig. 1. DGGE profiles of *nifH* amplicons from total DNA, extracted from guts of lagomorphs. Reamplification of bands 1, 12, 15, 16, 23, 27, 28, 44, 45, 53, 59, 60, 65, 67 failed. Bands 19–26, belonging to *O. mantchurica*, were excluded from discussion.

riety of diazotrophic inhabitants was detected in the cecum and colon of the pikas. Band patterns for all pika gut samples showed similar resolution trends with an average of 6 bands per lane. In total, 75 distinct bands from 13 lanes were excised, although reamplification was successful only for 61 bands from the lanes where the pika samples were resolved. Since the sequencing revealed that the bands with similar electrophoretic positions on polyacrylamide gel sometimes displayed different nucleotide sequence and could not be identified as the same organism, we have sequenced all the bands for which reamplification was successful. We obtained a total of 61 high-quality, chimera-free sequences for pikas (from 7 to 16 per animal).

The bands with the nucleotide sequences exhibiting over 95% similarity were designated as operational taxonomic units (OTU), which were numbered (Table 3). BLAST analysis revealed that most sequences demonstrated low identity with their closest relatives in the GenBank database, indicating presence of novel uncultured species. We excluded from further phylogenetic analysis the sequences obtained from the *Ochotona mantchurica* cecum, due to insufficient information for discussion. However, obtaining an amplification product with *nifH*-specific primers, as well as distinct bands after DGGE, make it possible to suggest that nitrogen-fixing organisms were present in the intestines of this particular Manchurian pika.

Phylogenetic reconstructions revealed that the NifH sequences obtained in the study were affiliated with two distinct clusters (Fig. 2). The first cluster containing 12 OTUs belonged to NifH Cluster I [22], comprising the organisms with 'conventional' Mo-containing *nifH* and some *vnfH,* which can be found in a wide variety of environments.These sequences are distantly related to NifH sequences of the cultured organisms *Sphingomonas* sp. KNUC167 (OTU 6) isolated from wild gramineous crops and *Bradyrhizobium* sp. TSA44 (OTU 11) found in rice paddy soil. The majority of the sequences were most similar to those of various uncultured bacteria detected in soil, plant rhizosphere, and other natural habitats, like hot springs [23] or soil under *Gymnostoma webbianum*, a plant which was shown to form nitrogen-fixing nodules [24]. Notably, organisms of this cluster mostly came from the colon, and both pika species examined in this study appeared to harbour them.

The second cluster contained 6 OTUs, which formed a common group with anaerobic organisms, distantly related to a well-known cellulose-degrading organism *Clostridium cellulovorans* 734B isolated from a batch methanogenic fermentation of poplar wood [25];

Table 3. Bands, comprising operational taxonomic units

OTU name, (number of bands)	Bands from cecum*	Bands from colon*
OTU 1, (2 bands)	21	30
OTU 2, (3 bands)	22	26, 32
OTU 3, (3 bands)	23	27, 33
OTU 4, (1 band)	No	29
OTU 5, (4 bands)	25, 83	37, 90
OTU 6, (5 bands)	47	52, 53, 54, 58
OTU 7, (1 band)	59	No
OTU 8, (1 band)	62	No
OTU 9, (4 bands)	49	55, 60, 65
OTU 10, (1 band)	No	66
OTU 11, (6 bands)	51	57, 67, 68, 70, 71
OTU 12, (1 band)	No	69
OTU 13, (5 bands)	50	56, 61, 73, 74
OTU 14, (3 bands)	75, 76, 77	No
OTU 15, (3 bands)	80	87, 92
OTU 16, (3 bands)	81	88, 93
OTU 17, (6 bands)	24, 82	28, 36, 89, 94
OTU 18, (1 band)	No	91

* As in Fig. 1.

to *Treponema* sp. Ru1 isolated from bovine gastrointestinal tract [26]; and *Fibrobacter succinogenes* S85, a cellulolytic bacterium present in the rumen of cattle [27]. Other representatives of this cluster are diazotrophic symbionts of various termite species: *Coptotermes gestroi*, *Mastotermes darwiniensis*, *Globitermes sulphureus* [28], *Reculitermes speratus* [29], and a xylophagous cockroach *Cryptocercus punctulatus*. This group of sequences belongs to the Cluster III according to Zehr et al., [22], consisting of *nifH* sequences from a diverse group of distantly related microorganisms many of which are strict anaerobes, like clostridia.

DISCUSSION

Symbiotic relationships between an animal and microorganisms that inhabit its gastrointestinal tract are among most common and interesting characteristics of a digestive system. These organisms include transient microorganisms and indigenous bacteria, which develop into relatively stable populations that can be species-specific [30]. While mammalian herbivores are known to host diverse microbial communities, microbial ecology of herbivorous rodents has received little attention thus far. To date, the most extensive effort to characterize microbial diversity of a mammalian gastrointestinal tract was made by Ley and coauthors and involved analysis of 16S rDNA sequences from fresh faeces of 59 species of non-human mammals [31].

Despite profound interest in the function and ecology of gut microbes, existing data on nitrogen-fixing bacteria of wild herbivore mammals are very limited.

Pikas are referred as hindgut fermenters, using the cecum for fermentation of food which is difficult to degrade by symbiotic bacteria, and colon for mixing the digested food by muscular contractions and absorbing water and nutrients. Microbial fermentation results mainly in production of volatile fatty acids, which are then absorbed by blood [32]. Protein for hindgut fermenters is synthesized by symbiotic microflora, excreted in the form of enriched soft feces and reingested.

Culture-independent analysis of the diazotrophic microbial community of the cecum and colon of the representatives of different *Ochotona* species provides an insight into diversity of microbes responsible for enriching the pika's diet with protein. The *nifH* gene fragment is a good tool for assessing the diversity of nitrogen-fixing organisms, since it is a conserved gene, although variable enough to distinguish between the species of host organisms [22]. Moreover, some researchers have shown that the *nifH* sequences of spirochaetes isolated from termites were very similar to the sequences obtained by direct amplification [29]. This fact makes it possible to suggest that at least some sequences derived by PCR analysis belong to genuine inhabitants of the pika cecum and colon, which are still waiting to be cultured.

The dendrogram we have constructed shows that the NifH sequences obtained from pika guts fall into two clusters. The Cluster I, comprising the sequences distantly related to 'conventional' nitrogenase of aerobic organisms from various natural environments, is mainly found in the colon, although some sequences from this cluster come from the cecum. The colon is known to contain large, fibrous food particles with low nutrition value, which leave the animal's intestine in the form of solid feces. Cultivable representatives of this cluster are aerobic organisms, mostly dwelling in soils, plant rhizosphere, and phyllosphere. Bacteria of the genus *Bradyrhizobium*, for instance, are known to form symbiotic nodules with legumes, thus providing these plants with fixed atmospheric nitrogen. Since pikas were shown to feed on legumes [33], we assume that *Bradyrhizobium* cells may get inside the animals with the plant material. Both species of pika appear to contain microorganisms of the Cluster I in their colons, and these organisms are most likely to enter their intestines with food, manage to escape digestion and leave with feces.

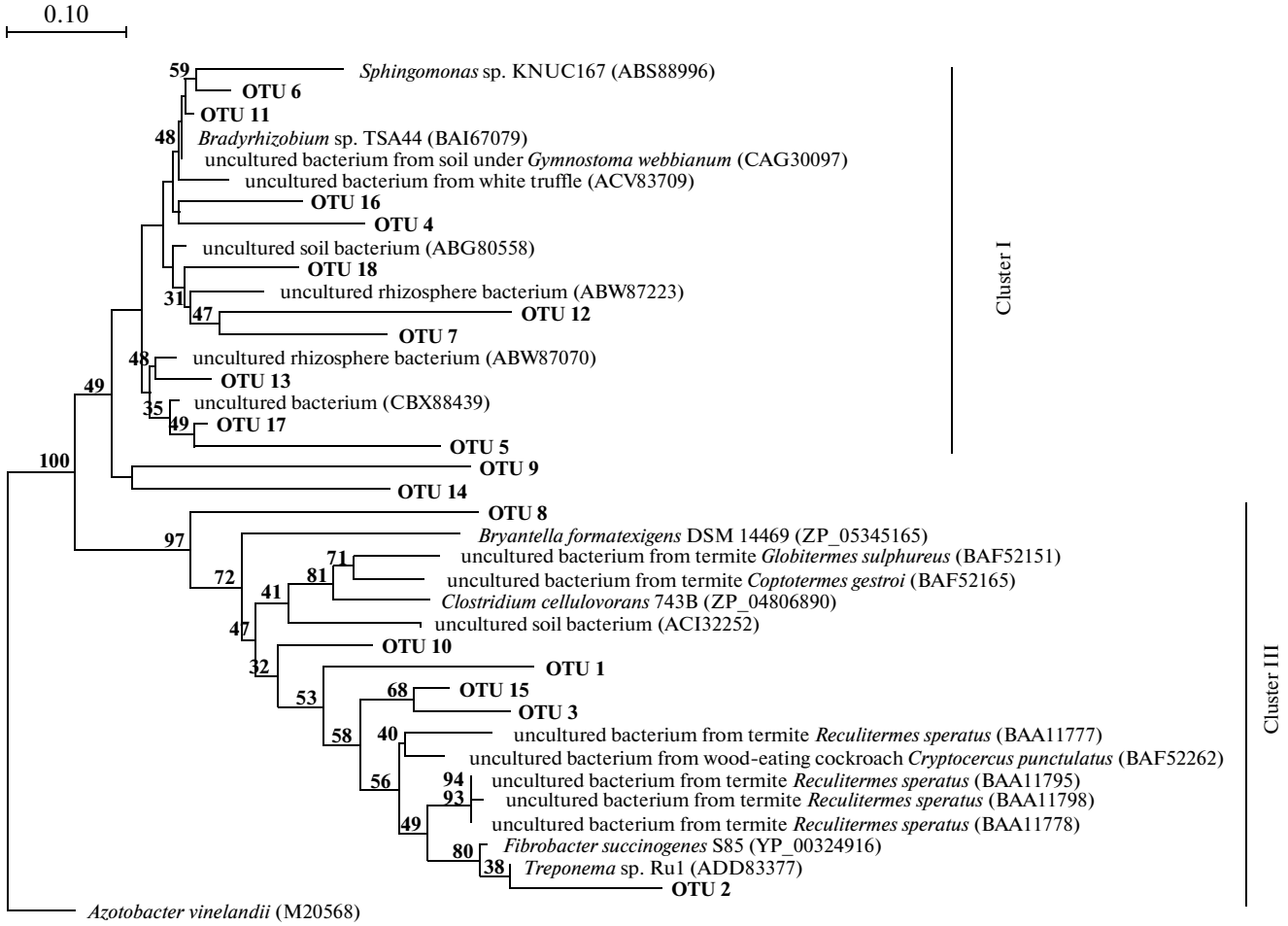

Fig. 2. A dendrogram, showing phylogenetic relations between amino acid sequences, deduced from the *nifH* gene fragments obtained in our study. The NifH of *Azotobacter vinelandii* served as an outgroup. The tree was constructed with the TREECON program (version 1.3b) using the neighbour-joining algorithm. Bootstrap values (100 data resamplings) ≥30% are shown. Bar, 0.10 substitutions per amino acid position. GenBank accession numbers are given in parentheses.

The Cluster III on the dendrogram consists of microbes quite distantly related to the organisms found in anaerobic environments and responsible for cellulose digestion, like *Treponema* sp. Ru1, isolated from bovine rumen [26] and most closely related to the cultured *Treponema zioleckii*, a fructan-utilizing species of the rumen treponemes [34]. This cluster also includes microorganisms detected in various termite species and in a wood-eating cockroach, as well as a representative of the genus *Clostridium*, *Clostridium cellulovorans* 743B, an anaerobe producing an extracellular enzyme complex able to degrade plant cell walls. *Clostridium* species were also found to inhabit the green iguana hindgut fermentation chamber [35]. These organisms come from the cecum and both parts of the pika colon. The cecum is a part of the gut of lagomorphs where fermentation takes place [30]; it is much bigger in lagomorphs than that in other non-ruminant herbivores, or foregut fermenters. The large cecum allows obtaining as much energy from nutrient-poor food as possible. The organisms belonging to this cluster are also found in both *Ochotona hyperborea* and *O. dauurica*. We suggest that these organisms are symbionts of pikas, dwelling in the fermenting part of their digestive system and responsible for enriching the low-nutrient food of these small lagomorphs with protein. Our data show that an organism from the colon of a female *O. dauurica* pika clusters with the group of anaerobic fermenting organisms. On the polyacrylamide gel, the band 47 representing this organism is near the band 43 from the same pika's cecum; this sequence fits into the same cluster. The male pika of the same species or pikas of other species didn't show any signs of similar organisms in the cecum or in the colon. The explanation for the presence of anaerobic fermenting organisms in the colon of both pika species is that, probably, fermentation occurs in the colon as well. Some authors suggest that the cecum is not the only place for fermentation in small hindgut fermenters, and that microbial fermentation also occurs in the small intestine and colon [32]. Some organisms from the cecum of both pika species were found in the clus-

ter containing mainly aerobic free-living organisms. These organisms probably originated from undigested food particles, which usually travel from the cecum to the colon in the cecum at the moment when the animals were sacrificed.

In order to see whether our suggestions were close to reality, Northern pikas were captured in 2009 and 2010 during fieldwork in the same region, and the *nifH* gene was amplified from same three parts of their intestines and resolved by DGGE. Northern pikas caught in 2009 and 2010 appeared to have similar diazotrophic inhabitants, grouping in both aerobic and anaerobic clusters according to the general trend we found for these animals. However, bands with the *nifH* fragments belonging to the animals caught in 2009 had slightly different location on the gel compared to those of the animals caught in 2010, which means these organisms had different GC content and were not the same ones. This fact can be possibly explained by the differences in the diet of investigated animals. Daurian pikas, both caught in 2010, showed almost identical patterns on the gel, except for the presence of an organism from the colon of a female pika in the fermenting anaerobic cluster, which allowed us to suggest that the colon might also be a place for fermentation in pikas (Fig. 1).

We also tried to find a relationship between the composition of diazotrophic gut community and pika species. Some researchers report that correlation exists between *nifH* gene phylogeny and termite taxonomic grouping [28]. Moreover, other studies report that overall bacterial community in the gut is significantly conserved within each genus of termites examined thus far [36]. However, we didn't find any significant difference between the diazotrophic gut communities of Northern pika and Daurian pika. No clusters or groups specific for any pika species have been detected. While not many organisms from the Daurian pika cecum could be found in the anaerobic cluster, compared to the organisms of the Northern pika cecum, more data are required to state that a group of anaerobic nitrogen-fixing dwellers of the Northern pika's cecum can be a species marker.

Results of the study expand our knowledge and understanding of the process of symbiotic nitrogen fixation in mammals, in small lagomorphs in particular. For the first time nitrogen-fixing inhabitants of intestines were studied in wild pikas. We have found that diazotrophic organisms from pika's intestines fall into two clusters. The first cluster is represented by a group of organisms related to aerobic bacteria inhabiting natural environments. The second cluster consists of the organisms, which may be symbiotic inhabitants of these small mammals, responsible for the fermentation of low-nutrient food these mammals eat. Further studies of nitrogen-fixing dwellers of lagomorph guts should focus on assessing the scale of this process, as well as on the understanding of the mechanisms of symbiotic nitrogen fixation in non-ruminant herbivores. Isolation and identification of pika gut symbionts will be a great advance in gut microbiology and a new step towards understanding host-microbe interactions.

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REFERENCES

1. Brett, C.T. and Waldron, K., *Physiology and Biochemistry of Plant Cell Walls*, London: Unwin Hyman, 1990, 194 p.
2. Van Soest, P.J., *Nutritional Ecology of the Ruminant*, 2nd ed., Ithaca, NY: Comstock Publishing Associates, 1994, 476 p.
3. Russell, J.B., *Rumen Microbiology and Its Role in Ruminant Nutrition,* New York: Ithaca, 2002, 119 p.
4. Clark, J.H., Klusmeyer, T.H., and Cameron, M.R., Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows, *J. of Dairy Science*, 1992, vol. 75, pp. 2304–2323.
5. Wood, T.G. and Johnson, R.A., The Biology, physiology and ecology of termites, in *Economic Impact and Control of Social Insects,* Vinson, S.B., Ed., New York: Praeger Publications, 1986, pp. 1–68.
6. Noirot, C., From Wood- to Humus-feeding: an important trend in termite evolution, in *Biology and Evolution of Social Insects*, Billen, J., Ed., Leuven: Leuven University Press, 1992, pp. 107–119.
7. Slaytor, K., Cellulose digestion in termites and cockroaches: what role do symbionts play?, *Comp. Biochem. Physiol.*, 1992, vol. 103B, pp. 775–784.
8. Benemann, J.R., Nitrogen fixation in termites, *Science*, 1973, vol. 181, pp. 164–165.
9. Noda, S., Ohkuma, M., Usami, R., Horikoshi, K., and Kudo, T., Culture independent characterization of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite *Neotermeskoshunensis*, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 4935–4942.
10. Brauman, A., Doré, J., Eggleton, P., Bignell, D., Breznak, J.A., and Kane, M.D., Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits, *FEMS Microbiol Ecol.*, 2001, vol. 35, pp. 27–36.
11. Hiracawa, H., Supplement: coprophagy in leporids and other mammalian herbivores, *Mammal Rev.*, 2002, vol. 32, pp. 150–152.
12. Eden, A., Coprophagy in the rabbit: origin of night feces, *Nature*, 1940, vol. 145, pp. 628–629.
13. Pshennikov, A.E., Alekseev, V.G., Koryakin, I.I., and Gnutov, D.Yu., Coprophagy in northern pika *Ochotona hyperborea* from Yakutia, *Zoologicheskii zhurnal*, 1990, vol. 69, pp. 106–114 (in Russian).

14. Langer, P., The Digestive tract and life history of small mammals, *Mammal Rev.*, 2002, vol. 32, pp. 107–131.

15. Braun, S.T., Proctor, L.M., Zani, S., Mellon, M.T., and Zehr, J.P., Molecular evidence for zooplankton-associated nitrogen-fixing anaerobes based on amplification of the *nifH* gene, *FEMS Microbiol. Ecol.*, 1999, vol. 28, pp. 273–279.

16. Formozov, N.A. and Baklushinskaya, I.Yu., Manchurian pika (*Ochotona mantchurica scorodumovi*) from the interfluve of the shilka and argun rivers: karyotype and problems of pika taxonomy in amurland and adjacent territories, *Zoologicheskii zhurnal*, 2011, vol. 90, pp. 490–497 (in Russian).

17. Poly, F., Monrozier, L.J., and Bally, R.,Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil, *Res. Microbiol.*, 2001, vol. 152, pp. 95–103.

18. Wartiainen, I., Eriksson, T., Zheng, W., and Rasmussen, U.,Variation in the active diazotrophic community in rice paddy – nifH PCR-DGGE analysis of rhizosphere and bulk soil, *Appl. Soil Ecol.,* 2008*,* vol. 39, pp. 65–75.

19. Muyzer, G., de Waal, E.C., and Uitterlinden, A.G., Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, *Appl. Environ. Microbiol.*, 1993, vol. 59, pp. 695–700.

20. Hall, T.A., BioEdit: a User-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucl. Acids Symp. Ser.*, 1999, vol. 41, pp. 95–98.

21. Van de Peer, Y. and De Wachter, Y., TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment, *Comput. Applic. Biosci.*, 1994, vol. 10, pp. 569–570.

22. Zehr, J.P., Jenkins, B.D., Short, S.M., and Steward, G.F., Nitrogenase gene diversity and microbial community structure: a crosssystem comparison, *Environ. Microbiol.*, 2003, vol. 5, pp. 539–554.

23. Hamilton, T.L., Boyd, E.S., and Peters, J.W. Environmental constraints underpin the distribution and phylogenetic diversity of *nifh* in the yellowstone Geothermal Complex, *Microb Ecol.*, 2011, vol. 61, pp. 860–870.

24. Hery, M., Philippot, L., Meriaux, E., Poly, F., Le Roux, X., and Navarro, E., Nickel mine spoils revegetation attempts: effect of pioneer plants on two functional bacterial communities involved in the n-cycle, *Environ. Microbiol.*, 2005, vol. 7, pp. 486–498.

25. Sleat, R., Mah, R., and Robinson, R., Isolation and Characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov., *Appl. Environ. Microbiol.*, 1984, vol. 48, pp. 88–93.

26. Evans, N., Brown, J., Murray, R., Getty, B., Birtles, R., Hart, C., and Carter, S., Characterization of novel bovine gastrointestinal tract *Treponema* isolates and comparison with bovine digital dermatitis treponemes, *Appl. Environ. Microbiol.*, 2011, vol. 77, pp. 138–147.

27. Stewart, C.S. and Flint, H.J., *Bacteroides* (*Fibrobacter*) *succinogenes*, a cellulolytic anaerobic bacterium from the gastrointestinal tract, *Appl. Microbiol. Biotechnol.*, 1989, vol. 30, pp. 433–439.

28. Yamada, A., Inoue, T., Noda, S., Hongoh, Y., and Ohkuma, M., Evolutionary trend of phylogenetic diversity of nitrogen fixation genes in the gut community of wood-feeding termites, *Mol. Ecol.*, 2007, vol. 16, pp. 3768–3777.

29. Ohkuma, M. and Kudo, T., Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus, Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 461–468.

30. Hume, I., Optimal digestive strategies in mammalian herbivores, *Physiol. Zool.*, 1989, vol. 62, pp. 1145–1163.

31. Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Birche, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., and Gordon, J.I., Evolution of mammals and their gut microbes, *Science*, 2008, vol. 320, pp. 1647–1651.

32. McBee, R., Hindgut fermentations in nonavian species, *J. Exp. Zool. Suppl.*, 1989, vol. 3, pp. 55–60.

33. Ritchie, M.E. and Tilman, D., Responses of legumes to herbivores and nutrients during succession on a nitrogen poor soil, *Ecology*, 1995, vol. 76, pp. 2648–2655.

34. Piknova, M., Guczynska, W., Miltko, R., Javorsky, P., Kasperowicz, A., Michalowski, T., and Pristas, P., *Treponema zioleckii* sp. nov., a novel fructan-utilizing species of rumen treponemes, *FEMS Microbiol. Lett.*, 2008, vol. 289, pp. 166–172.

35. McBee, R.H. and McBee, V., The hindgut fermentation in the green iguana, *Iguana iguana*, in *Iguanas of the World*, Burghardt, G.M. and Rand, A.S., Eds. Noyes, Park Ridge, New Jersey, 1983, pp. 77–83.

36. Hongoh, Y., Deevong, P., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Vongkaluang, C., Noparatnaraporn, N., and Kudo, T., Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host, *Appl. Environ. Microbiol.*, 2005, vol. 71, pp. 6590–6599.

37. Marusina, A.I., Boulygina, E.S., Kuznetsov, B.B., Tourova, T.P., Kravchenko, I.K., and Gal'chenko, V.F., A system of oligonucleotide primers for amplifying *nifH* genes from various taxonomic groups of prokaryotes, *Microbiology,* 2001, vol. 70, no. 1, pp. 86–91.

38. Sarita, S., Priefer, U.B., Prell, J., and Sharma, P.K.**,** Diversity of *nifH* gene amplified from rhizosphere soil DNA, *Curr. Sci.,* 2008, vol. 94, pp. 109–115.

39. Mehta, M.P., Butterfield, D.A., and Baross, J.A.*,* Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge, *Appl. Environ. Microbiol.*, 2003, vol. 69, pp. 960–970.

40. Rösch, C., Mergel A., and Bothe, H., Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.*, 2002, vol. 68, pp. 3818–3829.